The Immobilization of Spermatozoa by Freezing and Thawing and the Protective Action of Glycerol

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(*Received* 3 *June* 1954)

It was discovered by Polge, Smith & Parkes (1949) that spermatozoa could be frozen and thawed without loss of motility if glycerol was included in their suspending medium. Following this discovery the spermatozoa of the domestic fowl (Polge, 1951), of the bull (Polge & Rowson, 1952) and of the herring (Blaxter, 1953) have been preserved without loss of function for long periods in the frozen state. The importance of the practical applications of this method has been discussed (Polge & Parkes, 1952). The investigation of the protective action of glycerol was facilitated by the observation that red blood cells also were protected against the adverse effects of freezing and thawing by means of glycerol (Smith, 1950), and the nature of the damage occurring to red blood cells when they are frozen and thawed, and of the protection given by glycerol was then described (Lovelock, 1953*a*, *b*). Briefly, haemolysis by freezing and thawing is due to the destructive action of the concentrated salt solution to which the cells are exposed when water is removed as ice. In the presence of glycerol the electrolyte concentration at temperatures below the freezing point is sufficiently reduced to explain in full the protection afforded by glycerol.

The apparent simplicity of the processes causing the haemolysis of red blood cells by freezing and thawing, and of the protective action of glycerol, made it of some interest to observe the behaviour of other living cells on freezing and thawing. It seemed that this provisional explanation of the effects of freezing could best be tested using living cells with a wide range of sensitivity to hypertonic salt solutions. It also seemed desirable to use a criterion of damage more delicate than haemolysis.

Both of these requirements are met by the spermatozoa of different animals. Those of the rabbit are particularly sensitive to hypertonic salt solutions (Emmens, 1948), while those of the herring clearly will tolerate salt solutions at least as strong as sea water. In addition, the observation of the motility of spermatozoa, even if somewhat lacking in precision, provides a different and sensitive criterion for the assessment of freezing damage.

This paper records the effects of freezing and thawing upon the motility of the spermatozoa of the rabbit, the bull, the fowl and the herring. The modification of these effects by glycerol is also described.

METHODS

Semen. Rabbit and bull semen were collected using the techniques described by Macirone & Walton (1938) and Walton (1942). After collection, the semen contained in a glass test tube was placed in a 250 ml. glass beaker filled with water at 25°. The beaker was then placed in a refrigerator and left for 2 hr. to cool to 2°. This slow-cooling procedure was necessary because the spermatozoa of both the bull and the rabbit are sensitive to thermal shock. The semen was kept at 2° or in crushed ice at 0° until required for use, but was always used within 5 hr. of collection.

Fowl semen was collected by the method described by Burrows & Quinn (1935). After collection the semen was placed directly in a cold-room at 2°, and kept there until ready for use. Herring sperm were expressed directly from the gonads. These had been dissected from the herring and stored at 0° for not more than 2 days.

Suspending media. Before each experiment the semen was diluted with 9 vol. of the appropriate suspending medium at 0°. This was partly because the assessment of motility was easier with diluted semen, and partly to define the principal electrolytes of the cell suspension. The suspending media, which were kept at 0°, were:—rabbit: NaCl, 0·12 M; Na_2HPO_4, 0·01 M; KH_2PO_4, 0·01 M; glucose, 0·025 M; egg yolk 10%;—bull: trisodium citrate, 0·09 M; KH_2PO_4, 0·01 M; egg yolk 10%;—fowl: NaCl, 0·15 M; Na_2HPO_4, 0·01 M; KH_2PO_4, 0·01 M;—herring: sea water. When glycerol was used, it was included in the medium before mixing. Semen was mixed directly with media containing 0·5 M glycerol or less, but with higher glycerol concentrations the cells were left for 30 min. in a medium containing 0·5 M glycerol at 0°, and then diluted with medium containing sufficient glycerol to provide the desired final concentration. After every addition of glycerol the cells were left for 30 min. to ensure their permeation by the solute.

Measurement and maintenance of low temperatures. In all experiments temperatures were measured in terms of the e.m.f. generated by a calibrated copper–constantan thermocouple. Temperatures between 0 and −30° were obtained by means of a thermostatically controlled ethanol bath which was maintained at the required temperature within ±0·1°. Temperatures below −30° were obtained by means of a bath of ethanol (10 l.) contained in an open-necked Dewar flask and adjusted to the required temperature by the addition of solid CO_2. Under the conditions used the temperature remained constant during the 10 min. required for the experiments.

Assessment of motility. The cells were examined microscopically as a thin preparation between slide and cover glass, with a 16 mm. objective. The microscope was enclosed within a chamber kept at 37°, in which the preparation was left for 2 min. before examination. The motility of the spermatozoa under test was compared with the motility of the untreated controls kept at 0° and was expressed as an arbitrary score. Motility equal to that of the control was given a score of 10 and complete immobility a score of 0.

Procedure for exposing spermatozoa to concentrated salt solutions. Semen (0·1 ml.) was added to concentrated salt solution (0·3 ml.) contained in a small test tube at 0°. After 10 min. the mixture was diluted with 0·6 ml. of a mixture of the normal suspending medium and distilled water of such composition as would give a final concentration equal to that of the normal suspending medium. The diluted cell suspension was then warmed and examined for motility. The salt concentration above which a progressive fall in motility took place was recorded as that causing irreversible damage to the spermatozoa in 10 min. at 0°.

Procedure for freezing suspensions of spermatozoa. All experiments were made in flat-bottomed tubes (100 × 5 mm., wall thickness 1 mm.). The cell suspension (0·2 ml.) in one of these tubes was placed in a bath set at the required temperature. The usual procedure was to leave the suspension in the cold bath for 10 min. after freezing had taken place either spontaneously or by seeding with an ice crystal. With spermatozoa of the bull and of the rabbit a slightly different procedure was used in experiments below −10° to try to avoid thermal shock. The cells were first cooled to −10° and left there unfrozen for 10 min. (Supercooling of small volumes to this temperature is not difficult.) The unfrozen suspension was then transferred to a bath at the required temperature and there frozen and left for a further 10 min. After all experiments the cells were thawed by immersion in a water bath at 20°.

The choice of 10 min. for exposure to freezing was based upon pilot experiments and experience with other cells. These showed that destruction occurs rapidly during the first 3 min. after freezing but then slows considerably. In 10 min. most of the damage due to freezing has taken place.

RESULTS

Fig. 1 shows the depression of motility of the spermatozoa after freezing for 10 min. at temperatures between 0 and −45°. The effect of including various concentrations of glycerol in the suspending medium before freezing is also shown.

In Table 1 are the concentrations of the electrolytes used for suspending the cells, and the maximum concentrations which the cells will withstand at 10 min. at 0° without suffering some irreversible damage. Also shown are the freezing point of the latter solution, and the temperature below which the cells cannot be taken in the frozen state without suffering permanent damage. Corresponding values for human red blood cells are given for comparison.

DISCUSSION

Between 0 and −50° there is a critical region of temperature in which living cells perish if left for more than a few seconds. The precise bounds of this region for the human red blood cell are from −3 to −40° (Lovelock, 1953*a*). The protective action of glycerol is shown principally by its ability to reduce the extent of this critical region and, if sufficient is used, to remove it altogether. The results show that there are similar critical ranges of temperature in which spermatozoa suffer damage and that glycerol reduces the extent of these critical regions. The bounds of the critical regions and the temperatures at which the greatest damage takes place differ with the cells of different species.

The experimental results summarized in Table 1 show that the spermatozoa of the rabbit, bull and fowl are all more sensitive to freezing than the human red blood cell. On the other hand, herring spermatozoa are remarkably resistant to freezing; even in the absence of glycerol they are not damaged by freezing until the temperature falls below −6°, and are not destroyed completely until −15°.

The spermatozoa of the bull and of the rabbit are well known to be sensitive to thermal shock. This effect is described in detail in a recent review (Smith, 1954). The results (Fig. 1) show that for bull and rabbit spermatozoa no damage attributable to thermal shock took place down to −12°. Below this temperature the risk of damage by thermal shock increases as the temperature interval through which the cells are cooled becomes greater. At the lowest temperatures investigated, −20 to −30°, there is some chance that the observed death of cells may have been due to thermal shock as well as to the adverse effects of freezing and thawing. In the practical preservation of bull spermatozoa at −78° it is possible by using an adequate concentration of glycerol to cool sufficiently slowly to avoid damage by thermal shock. In the present experiments, however, which included freezing with low concentrations of glycerol, cooling at the rate found satisfactory for practical preservation would have been too slow. This is because the investigation of the lower limits of the critical region requires the passage of the cells through the rapidly destructive intermediate temperatures. Slow cooling through this unfavourable region would have completely obscured the effects of exposure to the lower temperatures.

The damage suffered by human red blood cells during freezing and thawing is due almost exclusively to the concentration of the electrolytes of the cell and their suspending medium as water is removed as ice. The results shown in Table 1 indicate that with all the spermatozoa tested the temperature at which damage first takes place is within 0·5° of the freezing point of a salt solution whose concentration is just sufficient to cause some irreversible damage. The concentration of electrolyte above which irreversible damage to the cell takes place appears to be characteristic for each

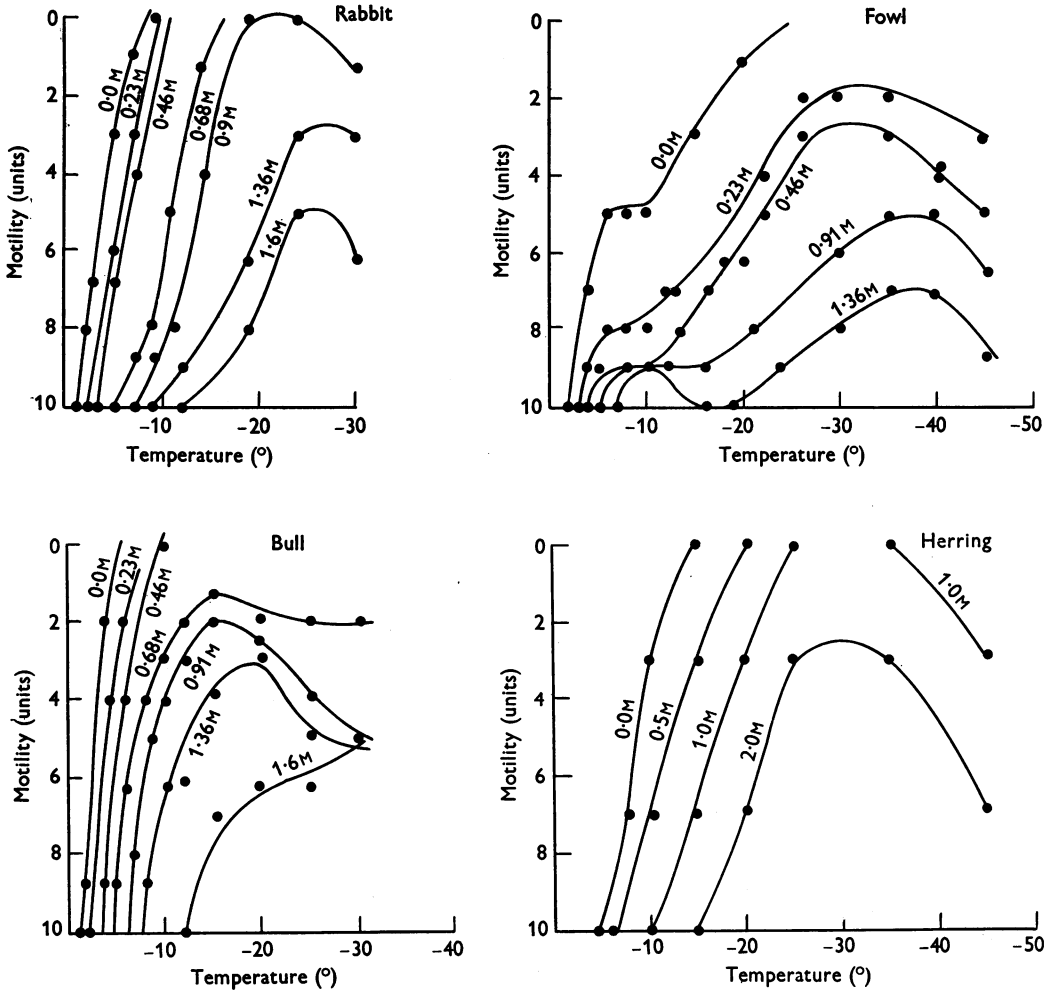

Fig. 1. The immobilization of spermatozoa of various species when frozen for 10 min. at the temperatures indicated. The cells were frozen in the media described in Table 1, and also in these media together with the concentrations of glycerol shown on each curve. The ordinates indicate the motility of the spermatozoa in the arbitrary units described in the text.

Table 1. *A comparison of the observed and calculated values for the temperature below which spermatozoa suffer irreversible damage when frozen*

Measurements were made of the temperature, ΔT_D, below which the cells suffered some irreversible damage, when frozen for 10 min. Measurements were also made of the electrolyte concentration, x_D, above which irreversible damage occurred when the cells were kept in it for 10 min. at 0° and then transferred back to their normal suspending medium. The calculated value of ΔT_D is the freezing point of a solution whose concentration is x_D.

Cell species	Suspending medium	Electrolyte concentration, x, of medium (M)	x_D (M)	ΔT_D Calculated (°)	ΔT_D Observed (°)
Rabbit spermatozoa	NaCl	0·12	0·32	−1·2	−1·0
Bull spermatozoa	Sodium citrate	0·09	0·18	−1·5	−1·5
Fowl spermatozoa	NaCl	0·15	0·35	−1·5	−2·0
Herring spermatozoa	Sea water	0·68	1·7	−6·2	−6·0
Human red blood cells	NaCl	0·15	0·9	−3·5	−3·0

type of cell. The herring spermatozoa which are normally exposed to sea water are very much more tolerant to increases in salinity than are the mammalian cells.

It follows from the colligative properties of solutions that the addition of a neutral solute such as glycerol will lower the concentration of salt in equilibrium with ice at any temperature below freezing. With the human red blood cell it has been shown that the protective action of glycerol is entirely due to this effect, and a simple relationship indicating the concentration of glycerol required to prevent damage to cells when they are frozen was deduced (Lovelock, 1954). This relationship can now be expressed

$$y = \frac{\Delta T_D - 2Kx_D}{Kx_D/x} = \left(\frac{\Delta T_D}{Kx_D} - 2\right) x,$$

where y = the concentration of glycerol required to prevent irreversible damage, x = the concentration of electrolyte in the suspending medium, nx (Lovelock, 1954) $= x_D =$ the electrolyte concentration at which damage first occurs, ΔT_D = the depression of the temperature below 0° at which damage first takes place, and K = a constant, indicating the observed depression of the freezing point of an aqueous solution by unit solute concentration.

It is clear that, since x_D is characteristic for each species of cell, the glycerol concentration required to protect a given species against damage by freezing will vary directly with the salt concentration of the medium used to suspend the cells before freezing.

Fig. 2 shows lines drawn according to this relationship using the values of x and x_D in Table 1 for each of the kinds of spermatozoa tested. The lines indicate the least concentrations of glycerol required by theory to protect the cells against irreversible damage on freezing to various temperatures. The points correspond to the observed glycerol concentrations found necessary in practice to protect the cells. Considering the inevitable lack of precision in the visual estimation of the motility of spermatozoa, the agreement between theory and practice is good for all of the spermatozoa tested. The experimental evidence offers strong support for the suggestion that the first cause of the death of spermatozoa on freezing is the increase of the concentration of their suspending medium to destructive levels. The protective action of glycerol can be explained completely in terms of its ability to prevent this increase.

On this basis it is clear that a relatively high concentration of glycerol is needed to protect herring spermatozoa, in spite of their great resistance to damage by freezing. With these cells their suspending medium, sea water, is already highly concentrated, and this presents a handicap to their successful preservation using glycerol. Herring spermatozoa will tolerate diluted sea water, and if they are frozen in this medium very much less glycerol is required than in sea water.

Red blood cells are protected by glycerol during freezing and thawing only when they are fully permeated by it. There is no benefit in preventing the electrolyte concentration of the suspending medium from rising if that of the cell interior is allowed to increase unchecked. No direct observations of the permeation of spermatozoa by glycerol

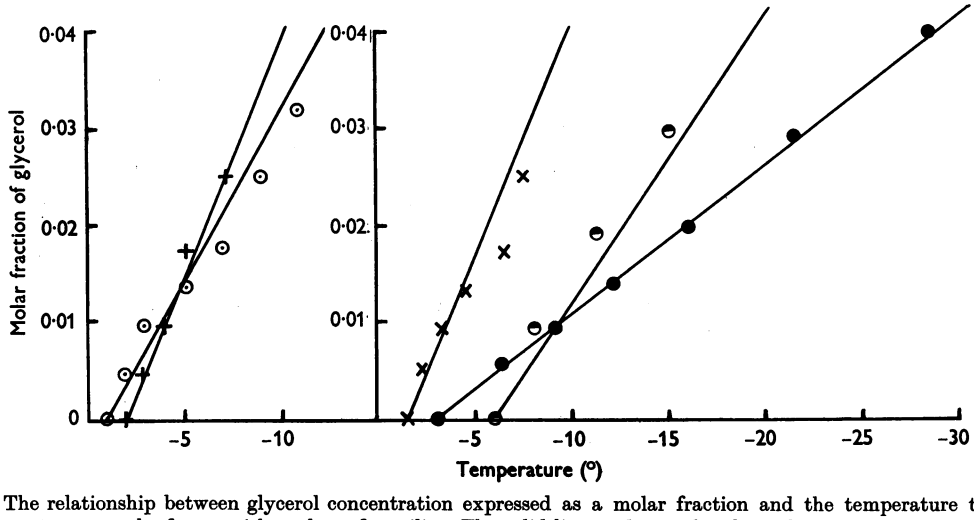

Fig. 2. The relationship between glycerol concentration expressed as a molar fraction and the temperature to which spermatozoa can be frozen without loss of motility. The solid lines indicate the glycerol concentrations required by theory, and the points the concentrations found necessary in practice. Spermatozoa: ⊙, rabbit; ×, bull; +, fowl; and ◓, herring. The theoretical line and experimental points for the human red blood cell, ●, using haemolysis as a criterion of damage, are also shown.

were made. All species of spermatozoa tested, including those highly sensitive to an increase in osmotic pressure, were fully motile in 2·0 M glycerol. It is most unlikely that cells impermeable to glycerol would retain motility in the presence of such an excessive external osmotic pressure, and the retention of motility in the presence of high concentrations of glycerol was taken as evidence that the cells were permeated by this solute. Other neutral solutes can to some extent protect spermatozoa against damage by freezing and thawing (Smith & Polge, 1950), but none is as effective as glycerol.

SUMMARY

1. The spermatozoa of the rabbit, the bull, the fowl and the herring were subjected to freezing and thawing in media containing various concentrations of glycerol, and observations made of the resulting changes in motility of the spermatozoa.

2. These observations indicated that the damage suffered by the spermatozoa during freezing and thawing was caused by their exposure to excessive concentrations of salt when water was removed as ice.

3. The concentration of electrolyte above which irreversible damage occurred was characteristic for the spermatozoa of each species tested. The protective action of glycerol was due to its ability to prevent the salt concentration rising above this level.

We are indebted to Dr A. S. Parkes, F.R.S., for his interest and advice. Our best thanks are due to Dr Audrey U. Smith who made freely available the results of her wide experience in freezing living cells. We should also like to express our thanks to Mr J. H. S. Blaxter, of the Marine Laboratory, Wood Street, Torry, Aberdeen, who supplied the herring gonads.

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A Study of the Metabolism of Phosphorus in Mammalian Red Cells

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(*Received* 24 *June* 1954)

It has been well established that orthophosphate containing radioactive phosphorus ([^{32}P-]orthophosphate) present in the plasma enters the mammalian red cell by way of an exchange process without a net increase in the phosphate concentration within the cell (Hahn & Hevesy, 1941; Gourley, 1952.) The radioactive phosphorus thus taken up by the cell is present within the cell not only as orthophosphate but also in the form of organophosphate esters. One can therefore visualize two aspects of the phosphate metabolism of the red cell: (1) the extracellular–intracellular exchange process involving the transport of [^{32}P]orthophosphate across the cell membrane, and (2) the intracellular partition of phosphorus amongst orthophosphate and various organic phosphate esters.

This paper will deal with the intracellular partition of phosphorus as studied by means of the addition of [^{32}P]orthophosphate to the extracellular medium. It will be shown that the relationship of the relative specific activities (R.S.A.) of the intracellular phosphate esters to each other and to orthophosphate suggests that (*a*) orthophosphate enters glycolysis by way of the glyceraldehyde 3-phosphate dehydrogenase reaction, and (*b*) that the intracellular inorganic phosphate fraction is not the main source of the phosphorus incorporated into the ester phosphate.

In order to lend further support to the foregoing conclusions, measurement of the rate of disappear-

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